

CalR Vignettes

Eight examples for visualizing and analyzing indirect calorimetry datasets using CalR templates

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Example 1: Two-group Analysis with ANCOVA

Quick Start: Load *CaIR* data file and *CaIR* Session file

The experiment in Example 1 will examine two groups of mice ($n=12$ WT, $10=KO$) maintained at 22°C for multiple days. The data in this example can best be analyzed with the Two-group Analysis Template. We will import our indirect calorimetry data from a standardized *CaIR* data file, which was previously saved after uploading 22 raw comma-separated value (CSV) files (The Columbus Instruments CLAMS Oxymax program exports one CSV file per mouse). In addition, we will make use of the optional feature to restore a previous workflow, avoiding the need to re-specify many inputs, by uploading a *CaIR* session file (Figure 1-1). With these examples, we present real and idealized sample data to illustrate the features of data analysis with *CaIR*.

Figure 1-1. The selections necessary to load data and group assignments for Example 1. Steps 3 through 5 are shown, with the key buttons outlined. Once the session file is uploaded, we observe that the group assignments are automatically designated. Proceed by clicking the “Go to plots” button.

Data Input: Standardized *CaIR* and Session files

1. From the *CaIR* home screen, select Templates > Two groups
2. By default, the Input tab (top) is selected
3. Under “Browse data CSV file(s)”, click on the “Browse...” button and locate and open the “Example1_CaIR.csv” file stored in the Example 1 folder
4. Under “Upload previous session inputs”, click “Browse...” and locate and open the “Example1_Session_NMR.csv” file stored in the Example 1 folder
5. Click the “Go to plots” button to be directly navigated to the “Time Plots” tab

From the “Time Plots” tab, we can begin visualizing the data. We can choose which metabolic variable to view by selecting the “Metabolic Variable” drop-down menu.

One of the advantages of the *CaIR* program is that it implements the most widely accepted method of analysis, which is the analysis of covariance (ANCOVA) where appropriate, and the generalized linear model (GLM) where ANCOVA is not appropriate. In each case, we utilize body mass as a covariate when modeling mass-dependent metabolic parameters. The data analysis is conducted over the time frame that is selected using the slider bar in the “Time Plots” tab.

Time Plots: Select metabolic variable to plot over time

1. Select the parameter to visualize: select Oxygen Consumption from the “Metabolic Variable” list. Time selections (hours 33-152) have been loaded from the session file
2. Click on the “Generate Plot” button (Figure 1-2)
3. Optional: groups colors can be specified by clicking on the boxes below the “Color of Group” label. Acceptable color inputs include words “blue”, HEX color codes “#0C0C96”, RGB color codes “rgb(12, 12, 150)” and HSL color codes “hsl(240, 92, 32)”

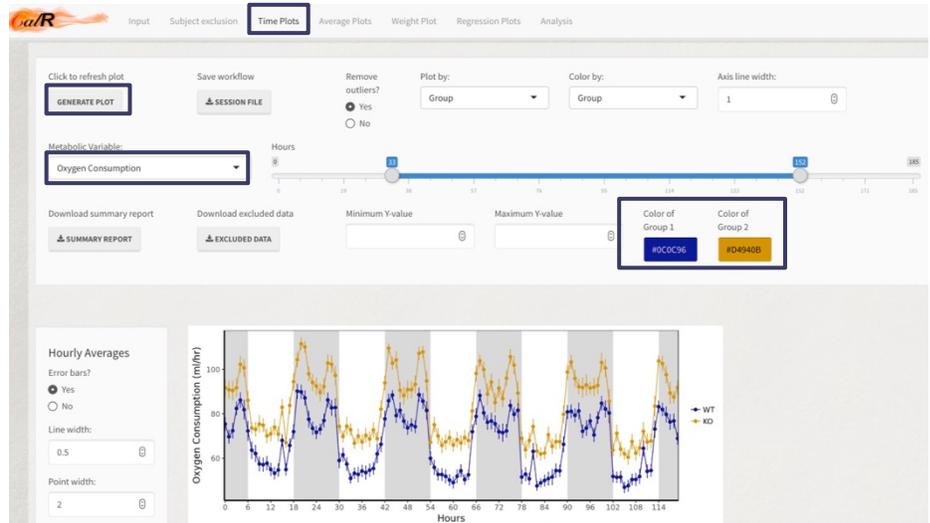


Figure 1-2. Time Plots in CalR. First, select a metabolic variable from the drop-down list and then click “Generate Plot.” Adjust time selection, inclusion of error bars, or group colors. After any changes are made to the inputs pertaining to the plots, it is necessary to press the “Generate Plot” button to apply changes, update the plot and the analyses. By default, hourly averages are plotted +/- SEM.

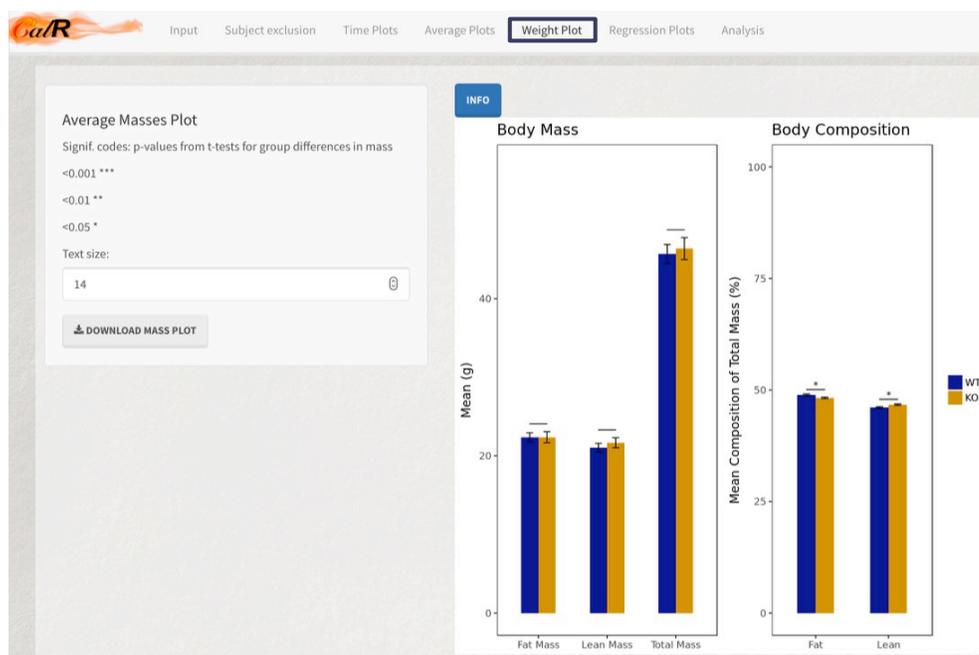
Average Plots: Daily and overall mean values by the time of day

Once a time range is specified in the Time Plots tab, we can navigate to the Average Plots tab (Figure 1-3). Here the mean value of the selected metabolic variable is plotted by day, group, and photoperiod in the top graph and overall values plotted by group and photoperiod in the bottom graph. Optionally, a data point for each animal is plotted over the bar graph. Data from the active analysis period can be downloaded for additional plotting and analysis. The data are provided as CSV files.



Figure 1-3. Average Plots in CalR. Top: Daily mean values plotted by group and photoperiod. Bottom: Overall mean plotted by group and photoperiod. The data selected in the Time Plots can be downloaded averaged either by hour, by day, or overall. Error bars represent +/- SEM.

Weight Plot: Compare group averages of body mass and body composition



Navigating to the “Weight Plot” tab, we compare the average total weights and percent body compositions between the groups. If significant differences exist, stars denote the level of significance as specified to the left of the plot (Figure 1-4).

Figure 1-4. Weight plot showing fat, lean, and total body masses in grams. Body composition is shown as a percentage of total mass.

Regression Plot: Associate energy expenditure with body mass in 2- and 3-dimensions

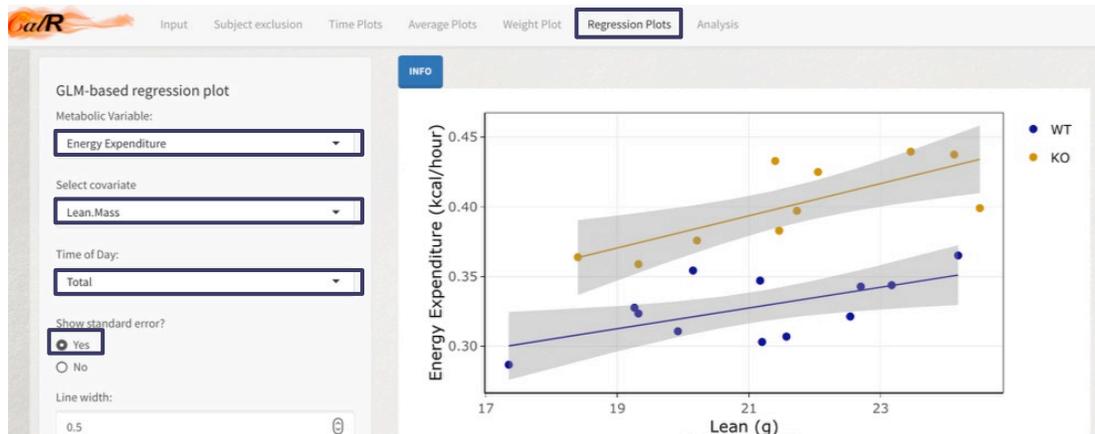
The regression plot provides a useful illustration of whether energy expenditure is dependent on mass, group (e.g., genotype), or both (Figure 1-5).

The significance test for “**Group effect**” is whether the two groups of interest, (here, WT and KO) are significantly different for the metabolic variable selected (here, energy expenditure). In this case, the group effect is significant and indicates that for a given total body mass, the KO subjects have higher energy expenditure (Figure 1-5A).

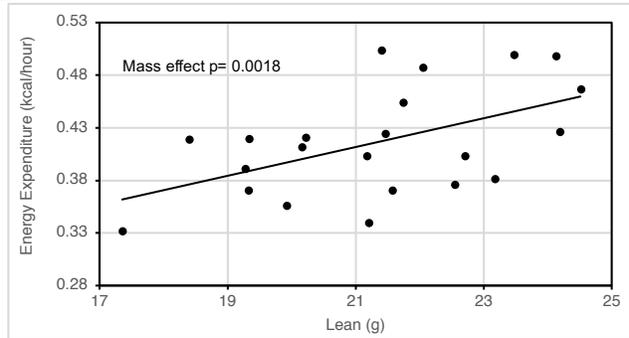
The significance test for “**Mass effect**” tells us if there is an association between the mass variable and metabolic variable selected among all animals in the study. In general, for mammals greater body mass is associated with greater energy expenditure. Here when we plot lean body mass against EE for all animals in the study, we see positive slopes for the mass effect and a p -value of <0.05 , demonstrating that on average our larger animals tend to expend more energy than smaller animals. (Figure 1-5B), When the mass effect is not significant or the slope is not positive, and the experiment is conducted at standard conditions (e.g., C57Bl/6 strain, chow diet, room temperature housing, sample sizes of at least six mice per group), the experimental interpretation may be valid, but should be approached with caution.

The “**Interaction effect**” tells us if there is a difference in the slopes of the two groups which would be attributed to the groups having different associations (non-parallel lines) between the mass and metabolic variable. The interaction effect is included in the model at first but if it is not significant ($p < 0.05$), CalR recalculates the model excluding the interaction effect and reports only the mass and group effects. If it is significant, the interpretation of the effects must be made with caution, as the estimated group difference is changing depending on the mass as demonstrated in Example 2.

A



B



C

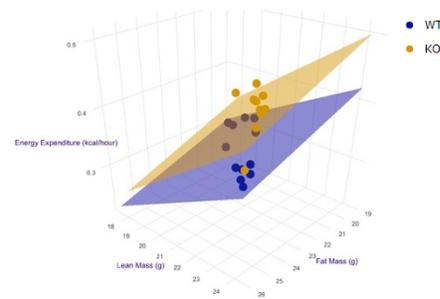


Figure 1-5 The Regression Plot visualizes the relationship between a metabolic variable (e.g. EE) and body mass or locomotor activity. (A) As displayed, the plot represents the association between subjects' energy expenditure, averaged over the dark photoperiods, and lean body mass. The *CalR* graphical user interface for the Regression Plots showing the selection for energy expenditure, lean mass, dark photoperiod and to display the standard error of the mean for each group. The GLM-based regression lines are shown for each group corresponding to the estimated EE at each mass and the shaded region represents the standard error. (B) The mass effect represents the correlation between the selected mass variable and metabolic variable, here EE vs LBM for all animals analyzed irrespective of group assignment. (C) When body composition data is available, 3D plots may be toggled, maneuvered, and saved. Clicking on the legend labels can toggle the appearance of points.

Validation of *CaIR*'s GLM-based regression model against the MMPC's Energy Expenditure Analysis tool

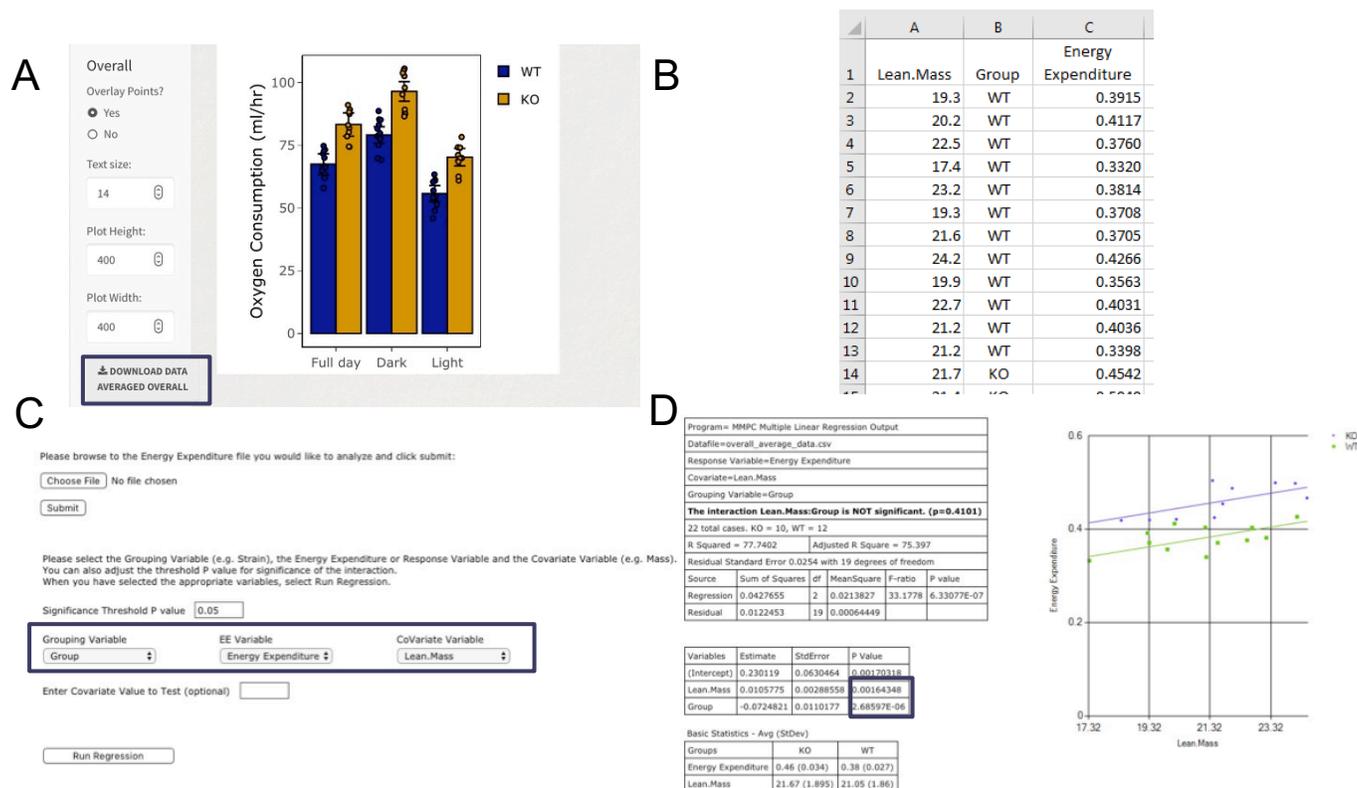


Figure 1-6. Validation of *CaIR* and MMPC analysis tool. (A) Extracting overall averages data from *CaIR*. (B) Filtering data by “Dark” time of day and including necessary columns. (C) Importing data into the MMPC web tool. (D) Obtaining output to compare with *CaIR*.

It is possible to extract data at various steps in the analysis produced by *CaIR*. For example, if we wish to further examine the overall average values for each mouse that are employed in the regression plots, these data are available to save as a CSV file. We can use this output in alternative programs such as the MMPC's Energy Expenditure Analysis tool found at <https://mmpc.org/shared/regression.aspx/>. Here we demonstrate the equivalence of these two tools (Figure 1-6). To obtain the overall average data from *CaIR*:

- Navigate back to the “Time Plots” tab
- Locate the button on the left of the overall bar plot labeled “Save data” (Figure 1-6A)

The downloaded data will contain the averaged values for each mouse in the period selected with the “Hours” slider input at the top of the page, which is the same data used in analyses within *CaIR*. Now we will prepare the data for importing into the MMPC web tool:

1. Open the saved data file, which should contain the name “overall_average_data.csv”, in your preferred spreadsheet software
2. Select only the data where the “Time of Day” value is “Dark”
3. Delete all columns that will not be needed by the MMPC web tool, only keeping “Lean.Mass”, “Group”, and “Energy Expenditure”. Save the file
4. In your web browser, navigate to <https://mmpc.org/shared/regression.aspx/>

5. Locate the button labeled “Choose file” and use it to select the file you just saved
6. Press “Submit”
7. More input options should appear now; for “Grouping Variable” select “Group”, for “EE Variable” select “Energy Expenditure”, and for “Covariate Variable” select “Lean.Mass”
8. Press “Run regression”

We find that the main plot produced by the MMPC tool (Figure 1-6D) strongly resembles the one produced in *CalR* (Figure 1-5A) and the *p*-values resemble those in the analysis tab of *CalR* (Figure 1-6). *CalR* reports the group effect for EE in the dark photoperiod as <0.001. When the complete analysis is downloaded from *CalR*, the exact figure is reported as $p=0.0018$. The MMPC web tool reports $p=0.0017$, a negligible difference of 0.0001.

Analysis with ANCOVA and ANOVA

CalR will examine the data for significant interaction effects between mass and mass-dependent metabolic variables. If an interaction is detected for any metabolic variable, then the GLM results are displayed. If the interaction effect is absent, the model is re-run and the ANCOVA results are displayed. Here, no significant interaction effect is observed (since $p>0.05$) and the results of the ANCOVA are returned (Figure 1-6).

To obtain GLM results:

1. Navigate to the “Analysis” tab and the table will populate automatically
2. Keep the default selection for “Select covariate” so that lean mass is the covariate in the GLMs
3. To get more information regarding the GLM used to estimate oxygen consumption, we can download summary

Model Significance

The tables display p-values corresponding to group effect (ANOVA) along with the mass and interaction effects as necessary (GLM)

Signif. codes: <0.001 ***, <0.01 **, <0.05 *

Select covariate

Total.Mass

ANALYSIS TABLES

Supplemental statistics for model

Metabolic Variable:

Oxygen Consumption

Select Time of Day

Total

Download supplemental model summary tables for:

SELECTED VARIABLE

ALL VARIABLES

Effect	Full Day			Light			Dark		
	Mass	Group	Interaction	Mass	Group	Interaction	Mass	Group	Interaction
Oxygen Consumption (ml/hr)	<0.001 ***	<0.001 ***		0.0020 **	<0.001 ***		0.0034 **	<0.001 ***	
Carbon Dioxide Production (ml/hr)	0.0011 **	<0.001 ***		0.0029 **	<0.001 ***		0.0051 **	<0.001 ***	
Energy Expenditure (kcal/hour)	<0.001 ***	<0.001 ***		0.0011 **	<0.001 ***		0.0031 **	<0.001 ***	
Hourly Food Consumed (g)	0.7839	<0.001 ***		0.4180	0.0089 **		0.8545	<0.001 ***	
Total Food Consumed (g)	0.5887	0.3426		0.6112	0.3508		0.5678	0.3350	

Effect	Full Day		Light	Dark
	Group	Group	Group	Group
Respiratory Exchange Ratio	0.0129 *	0.0237 *	0.0077 **	
Locomotor Activity (beam breaks)	0.0040 **	0.0053 **	0.0038 **	
Ambulatory Activity (beam breaks)	0.0567	0.1453	0.0362 *	

Figure 1-7. Analysis results: Three ANCOVA/GLM models are produced for each metabolic parameter, for 24-hour, light and dark photoperiods in the top table. This table displays *p*-values corresponding to the model coefficients for group effect (ANOVA) along with the mass and interaction effects as necessary (GLM). In the GLM models, the interaction effects were tested, found to be insignificant, and removed from the models. The bottom table displays the results of ANOVA analyses on mass-independent measurements.

statistics by clicking the “Selected Variable” button under “Download complete analyses tables for:”

Return to the Time Plots tab and click the “Download report” button to generate a PDF file with the hourly/daily plots, overall plots, weight plot, regression plot, and analysis. In this example experiment, among other findings, there was a significantly increased energy expenditure, VO_2 , VCO_2 and hourly food intake in the KO group for all time periods.

Interpretation

In this experiment, we have two groups of mice with no significant difference in body weights. The KO group has a greater metabolic rate and food intake with lower levels of locomotor activity. The KO mice also display a consistently lower RER,

suggesting greater levels of lipid oxidation. We would interpret this model as the KO mice maintain a higher metabolic rate due to the greater efficiency of lipid oxidation to produce energy.

Example 2: Two-group analysis using the generalized linear model when group and mass variables interact

In this example, we are examining two groups of mice (n=18 per group) maintained at thermoneutrality for five days. This example will illustrate the interaction effect when it appears significant. It will also demonstrate the use of the GLM-based regression plot to visualize the interaction effect. For this example, we will again use the Two Group Analysis template. The data we will use are stored in a *CalR* file.

Figure 2-1. Loading *CalR* data and Session files. Group labels, body weights, body composition data, and selections are automatically populated from the *CalR* Session file.

Data Input: *CalR* and Session files

1. Under “Browse data CSV file(s)”, click on the “Browse...” button.
2. Locate and open the Example2_ *CalR*.CSV file stored in the Example 2 folder
3. Under “Upload previous session inputs”, click “Browse...” and locate and open the “Example2_Session_NMR.CSV” file stored in the Example 2 folder
4. Click the “Go to plots” button to be directly navigated to the “Time Plots” tab

Time Plots: View by group or individual and detect outliers automatically

In this example, we can observe points where the energy expenditure readings are not accurate due to momentarily opening the cage to feed the mice during experimental data collection. When examined by *CalR*, these values are found to exceed 3 standard deviations from the group mean over the time period selected. If Remove Outliers is active, these values will be excluded from the plots and analyses and will be represented in the excluded data file. If Remove Outliers is off, then these values will be included in the plots and analyses (Figure 2-2).

Now that we have loaded the data, we will visualize the “remove outlier” function:

1. Start by setting the metabolic variable to “Energy Expenditure”
2. Set the radio button titled “Remove outliers?” to “No”
3. Under the “Plot by group or individual?” drop-down menu, choose “Individual”
4. Upon clicking “Generate plot” and seeing the default time plot, we can identify strange values during the first day and last day attributed to improperly sealed chambers
5. Set the “Remove outliers?” radio button back to “Yes” and observe the abnormal reading become detected and removed by *CalR*
6. Click on Download Excluded to generate a report which includes all automatically removed data

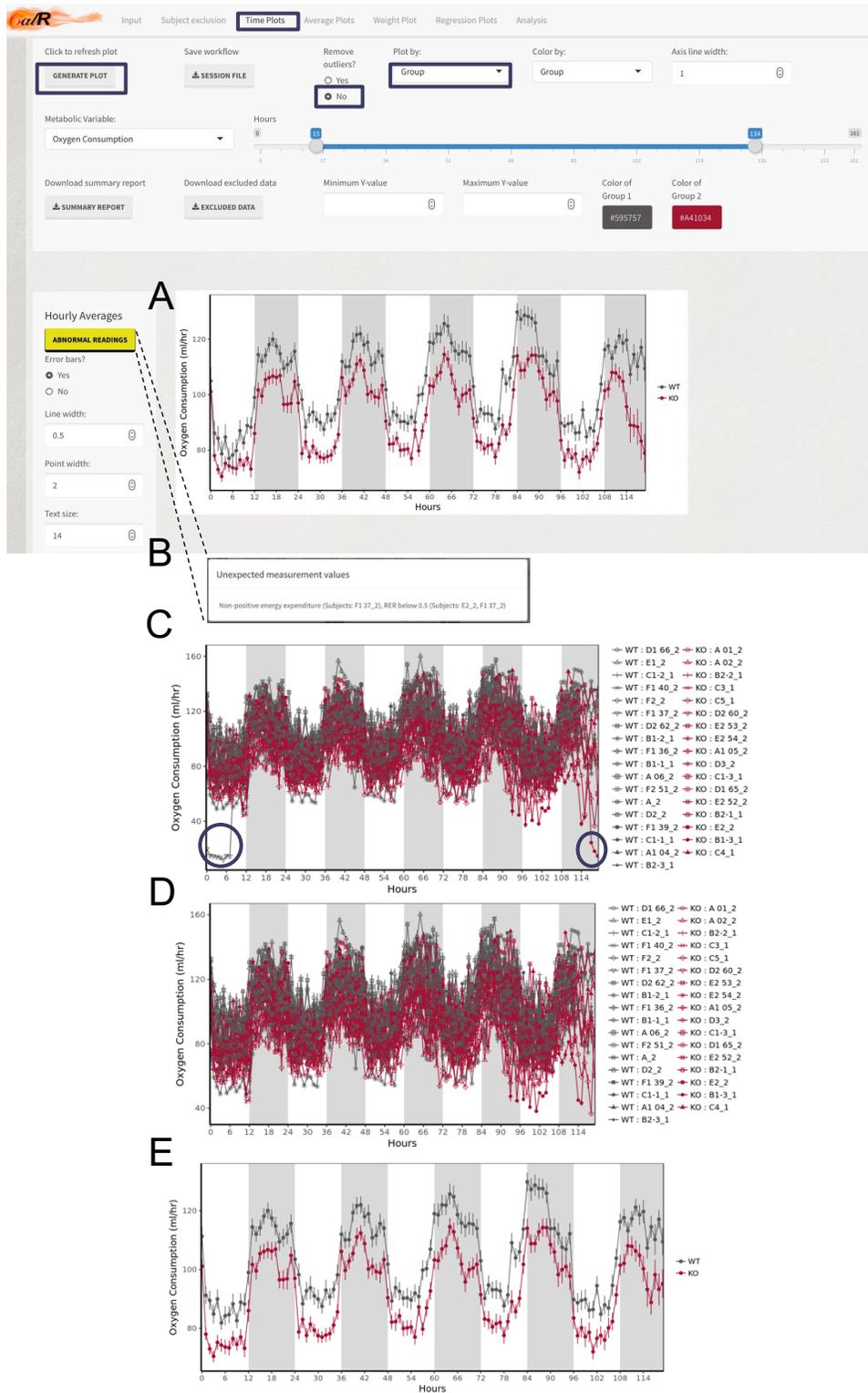


Figure 2-2. Illustration of remove outlier function. By default, the remove outlier option is set to off. When plotting by group averages (A), an Abnormal Readings button appears. When clicked, the abnormal readings button reveals multiple messages (B). Plotting by individual animals (C) before or (D) after auto-outlier removal. (E) Plotting by group following outlier removal. Excluded data from outlier detection is stored in a downloadable “Excluded data” file.

Regression Plots: Generalized linear model when there is an interaction effect

In this example, we have a phenomenon where there appears to be a group effect on energy expenditure, but a closer look will show that there is a difference in how mass is contributing to the group effect through an interaction.

1. Navigate to the “Regression Plots” tab
2. Use the “Select covariate” drop-down menu to choose “Lean.Mass”
3. The plot will automatically show the GLM-generated regression lines and will provide the p -values corresponding to the difference effects in the model below the plot
4. Optional: toggle plotting the standard error of the mean values by selecting the “Yes” radio button under “Show standard error?”
5. You may always click on the “Info button to get more information regarding the plot being shown, but if a yellow “Caution!” button appears next to it, then either i) there is no observable mass effect, or ii) a significant interaction effect is detected between mass and group (e.g. genotype). *CalR* does not know whether standard experimental conditions are being studied and these may be acceptable outcomes. The Caution indicator serves as a reminder that the experimental interpretation may need to be carefully considered (Figure 2-3)



Figure 2-3. Illustration of regression plot containing a significant interaction effect. The GLM is calculated based on the selected covariate and time of day from the inputs on the left. Toggling the standard error displays shaded regions with the group mean +/- the standard error.

Analysis: GLM and ANOVA

After visualizing the data, we will want to see the complete analysis (Figure 2-4).

1. Navigate to the “Analysis” tab
2. Select “Lean.Mass” as a covariate

Each metabolic variable is analyzed in a distinct model. Metabolic variables in the GLM table (top) include the selected mass covariate in the analysis. Variables in the ANOVA table (bottom) do not include the mass covariate. Furthermore, the GLM models independently test for an interaction effect for each parameter. If the interaction effect is nominally significant ($p < 0.05$) then it is kept and reported, otherwise, the model is re-run without the interaction term and the entry remains blank.

Model Significance

The tables display p-values corresponding to group effect (ANOVA) along with the mass and interaction effects as necessary (GLM)

Signif. codes: <0.001 ***, <0.01 **, <0.05 *

Select covariate

Lean.Mass

[ANALYSIS TABLES](#)

Effect	GLM								
	Full Day			Light			Dark		
	Mass	Group	Interaction	Mass	Group	Interaction	Mass	Group	Interaction
Oxygen Consumption (ml/hr)	0.0038 **	0.0530	0.0185 *	<0.001 ***	0.0127 *	0.0034 **	0.1168	0.0014 **	
Carbon Dioxide Production (ml/hr)	0.0039 **	0.0092 **	0.0025 **	<0.001 ***	0.0021 **	<0.001 ***	0.0177 *	0.0348 *	0.0116 *
Energy Expenditure (kcal/hour)	0.0021 **	0.0316 *	0.0099 **	<0.001 ***	0.0068 **	0.0016 **	0.0147 *	0.1092	0.0453 *
Hourly Food Consumed (g)	0.0575	0.0899		0.3327	0.1910		0.0649	0.1699	
Total Food Consumed (g)	0.9487	0.3832		0.9710	0.3681		0.9375	0.3926	

Effect	ANOVA		
	Full Day	Light	Dark
	Group	Group	Group
Respiratory Exchange Ratio	0.0068 **	0.0127 *	0.0054 **
Locomotor Activity (beam breaks)	0.0287 *	0.0488 *	0.0203 *

Figure 2-4. Analysis tables showing significant group x lean mass interaction effect for modeling EE. We can see that VO₂, VCO₂ and EE all have a significant interaction effect in all times of day (full day, light, and dark).

Locomotor Activity Regression Plots

Our analysis finds a significant group effect for locomotor activity during all photoperiods. Viewing locomotor activity in the Time plots and Average plots suggests greater locomotor activity in KO mice. From this analysis, we may also speculate whether the greater locomotor activity in the KO group contributes meaningfully to EE. Under the Regression Plots tab, we can plot the relationship between EE and locomotor activity. Like the EE vs mass regression plot, the EE vs locomotor activity regression plot will examine the overall data set (without consideration of group assignment) for a significant “Activity Effect.” Investigation of this topic was nicely described in Virtue, Even and Vidal-Puig, Cell Metabolism 2012 suggesting that under non-thermoneutral conditions, changes in activity do not make a large contribution to total daily EE. The data from this example is consistent with that conclusion. We examine mice housed at 23°C which yield an Activity Effect p -value of 0.858. Our KO mice have decreased overall EE despite increased locomotor activity (Figure 2-5).

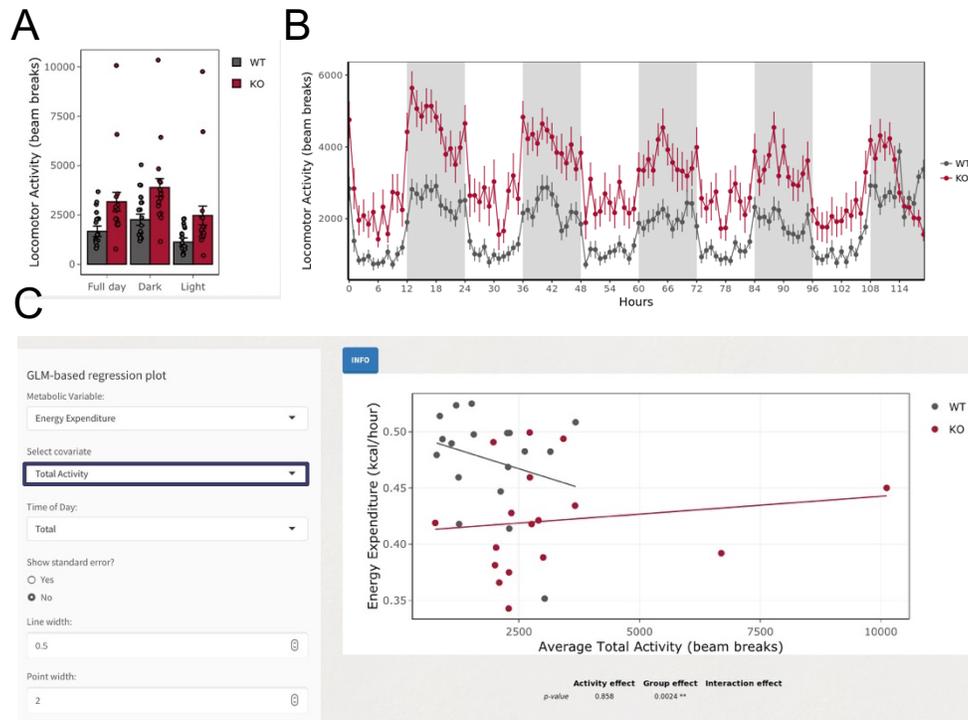


Figure 2-5. Illustration of a locomotor activity vs EE regression plot. (A) Overall increased mean locomotor activity in KO animals. (B) Locomotor activity vs time. (C) EE vs locomotor activity can be viewed in the Regression Plots tab. The Activity Effect is a measure of significance whether locomotor activity correlates with the selected metabolic variable.

Interpretation

We observe a significant interaction effect between the genotype (group) and lean mass (covariate) for VO_2 , VCO_2 , and EE. The regression plot for EE vs mass shows that KO mice do not appear to expend more energy per unit increase in lean mass. In contrast, WT mice do expend more energy per unit increase in lean mass, as we typically observe under these conditions (room temperature, standard low-fat chow diet, C57Bl/6J strain). The analysis also shows that RER and locomotor activity have significant group effects. The KO mice in this example have a decreased metabolic rate despite similar levels of food intake and greater activity.

Example 3: Two-group acute response analysis

In this example, we will utilize the template for an experiment during which the subjects in two groups were given a treatment and the acute response was observed. For this example, we will use the Two Group Acute Response Template. This

template is distinct from the standard two-group response as here we can view the acute response to the treatment when our time range is twelve hours or less, which is when *CaI/R* will automatically test for group differences at each hour. We will import a *CaI/R* and Session file and select the first value in the time range as the time of treatment (Figure 3-1).

Data Input: Standardized *CaI/R* file and weight template

1. Under “Browse data CSV file(s)”, click on the “Browse...” button and open the “Example3_*CaI/R*.csv” file stored in the Example 3 folder
2. Under “Upload previous session inputs”, click “Browse...” and open the “Example3_Session_NMR.CSV” file stored in the Example 3 folder
3. Click the “Go to plots” button

Time Plots: Select time range, normalization method, and mass covariate

1. The slider bar at the top of the page should be set to display the first value at hour 143 and second value at 153
2. Click on “View absolute or percent changes?” and select absolute
3. Click on “Select Covariate” and choose Lean.Mass
4. Click on “Generate plot” button at the top left corner of the page (Figure 3-2)

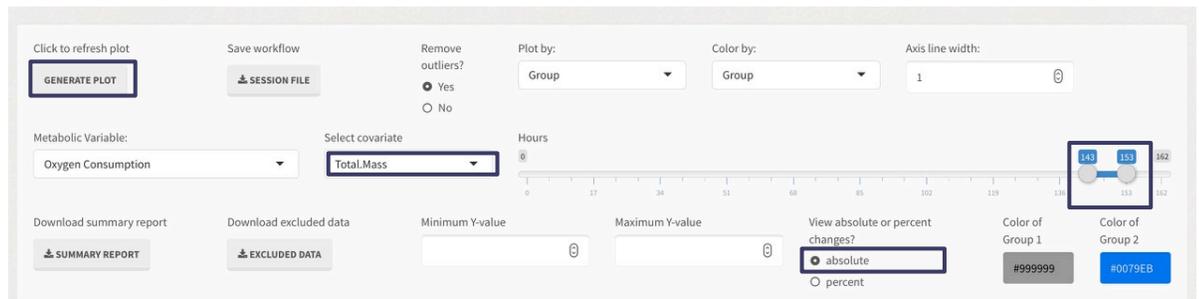


Figure 3-1. Plotting the two-group acute response example. The acute response analysis is active only for periods of 12 hours or fewer. In this template, the average change from initial value can be represented in a second plot by absolute difference or percent difference under “View absolute or percent changes?” A mass variable is selected only within the Time Plot tab in this template.

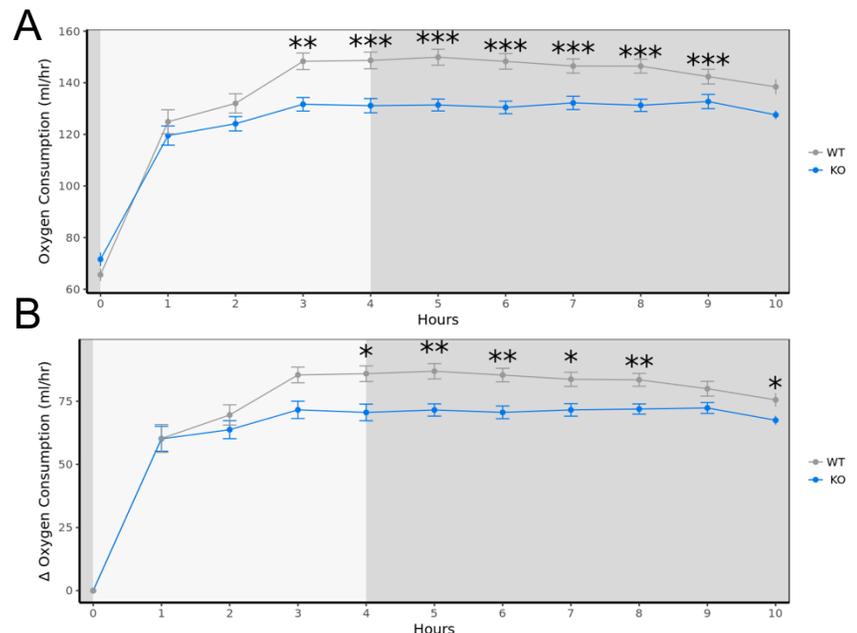


Figure 3-2. Example of annotations demonstrating significant differences within the acute response template. In this example two groups of mice were examined for their acute response to a beta adrenergic agonist which stimulates metabolic rate. On the time plots shown, we observe the acute response by (A) the mean VO_2 at each hour and (B) the average change in VO_2 from hour 0 at each subsequent hour. Stars represent significance at each point by GLM. Error bars represent SEM. *, $p < 0.05$; **, $p < 0.01$; *, $p < 0.001$.**

Analysis: ANCOVA at each hour after treatment

The comparisons at each time point in Figure 3-2 are GLM analyses with the selected mass covariate included in the model. If a significant interaction exists, then the ANCOVA results will be displayed. There is no correction for multiple comparisons performed in this module. If appropriate, the user should adjust these results accordingly.

1. To view the analysis of covariance, click on the “Analysis” tab
2. Change the “Select covariate” input to be “Lean.Mass”

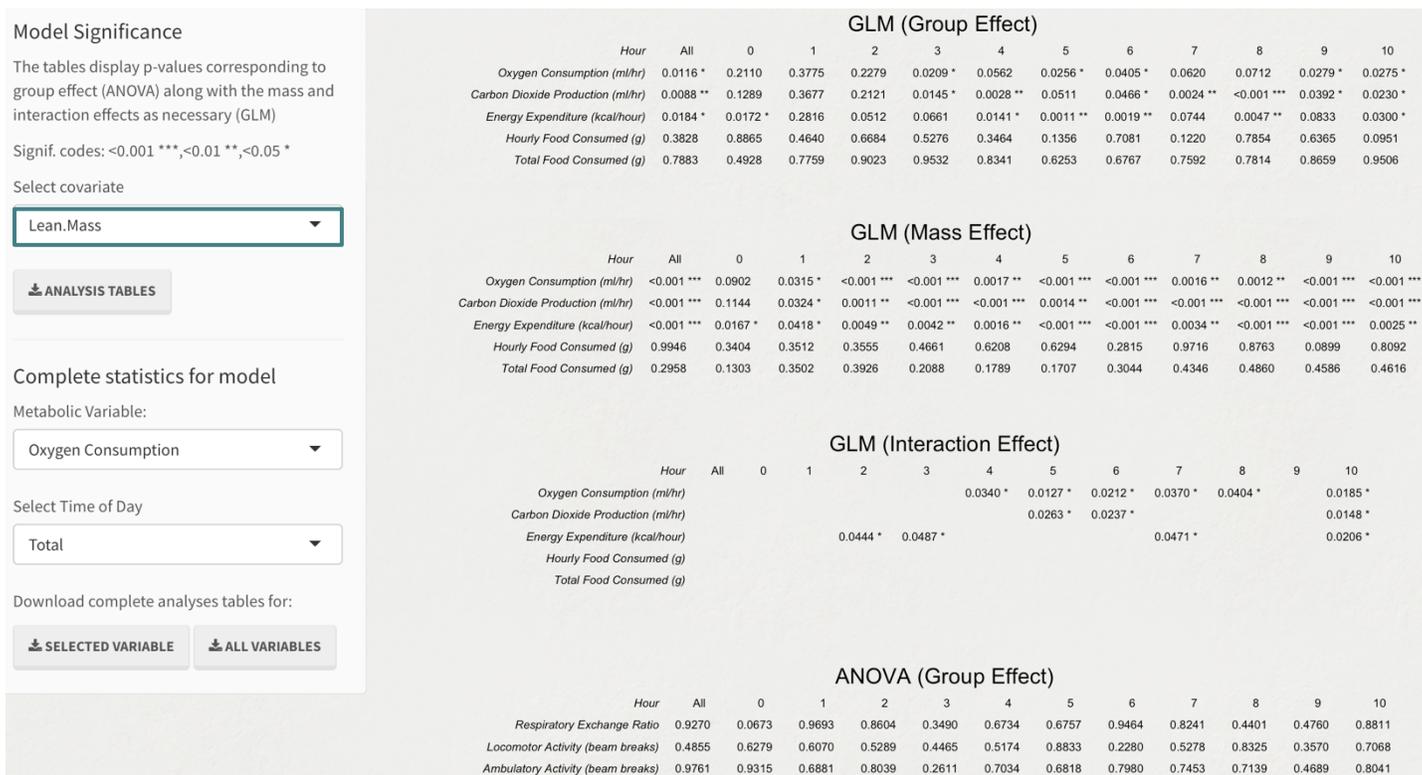


Figure 3-3. Complete hourly and overall analysis results for the acute response template. For each metabolic parameter, we see *p*-values from the 10 hours together under the column labeled “All” and *p*-values at each of the 10 hours independently analyzed by ANCOVA/GLM or ANOVA. The hours in this table correspond to the plots displayed in Figure 3-2.

Interpretation

The GLM (Group Effect) table indicates an overall VO_2 , VCO_2 , and EE differences over the 10-hour region specified (All); furthermore, we can observe specifically at which hours the differences occur between genotypes.

In the GLM (Mass Effect) table, we can observe that the mass effect with the lean body masses of the mice consistently appear as an important predictor of energy expenditure, but not for the amount of food consumed. In other words, here we observe mice with more lean mass expend more energy, but do not necessarily consume more food. Considering the non-standard conditions of the acute treatment which introduce a mild stress response, this is not a cause for concern, although under standard conditions we would expect that heavier mice would eat more to maintain their greater EE.

The GLM (Interaction Effect) table indicates that there is a significant interaction effect between mass and metabolic variables at certain hours.

Example 4: Analysis of three ordered groups with manual subject exclusion

In this example, we will examine a data set in which one animal had to be removed from its cage to prevent it from experiencing distress. After removal, data continues to be collected from the empty cage and is averaged with data from the other animals in the group. We will demonstrate how to identify the subject and time when the animal was removed and manually select the hour at which to start excluding said subject from the analysis (Figure 4-1). This analysis was performed using the Three-group: ordered template.

We use this template when our groups are factored so that they form a series. Examples include wild type, heterozygous, knockout; or administration of a compound in doses 0, 1, 2 mg/kg. When we use a template for more than two groups, *CalR* produces post hoc plots representing the pairwise comparisons with the mean difference at a 95% confidence interval.

Data Input: *CalR* and Session files

1. Under “Browse data CSV file(s)”, click on the “Browse...” button
2. Locate and open the Example4_ *CalR*.CSV file stored in the Example 4 folder
3. Under “Upload previous session inputs”, click “Browse...” and locate and open the “Example4_Session_NMR.CSV” file stored in the Example 4 folder
4. Click the “Go to plots” button to be directly navigated to the “Time Plots” tab

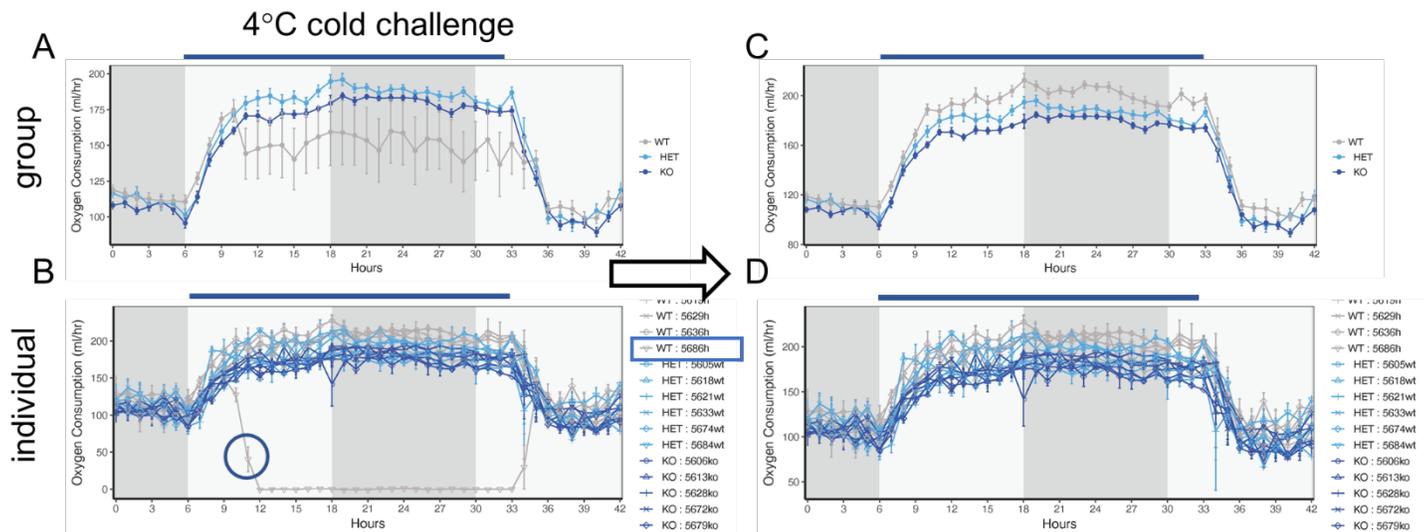


Figure 4-1. Three group ordered analysis with manual subject removal. (A) Data visualization by group reveals large variation starting at hour 10. This corresponds to an animal which was removed from the indirect calorimeter at hour 12 after a failure to adapt to the cold challenge. (B) Viewing the data by individual shows which animal was removed. (C-D) After subject exclusion for this one cage, the data can be appropriately analyzed. Blue bar corresponds to time of cold challenge.

Time Plots: Determine time for manual subject exclusion

1. Use the slider bar at the top of the page to set the first value to 132 and the second value to 174
2. Use the “Metabolic variable” drop-down menu to select “Oxygen Consumption”
3. Click on “Generate plot” button at the top left corner of the page
4. To get a better look, use the “Plot by group or individual?” drop-down menu and select “Individual”, also select “No” under “Error bars”
5. Click on Generate Plot.
6. Next, click on the Regression Plots tab and hover over the point at the bottom of the graph (Figure 4-2A)

Manual Subject Exclusion: Starting hour and annotation

1. Navigate to the “Subject exclusion” tab
2. Scroll down subject 5686h and enter 142, the hour to start the exclusion, into the numeric input field below the subject’s name (Figure 4-2B)
3. In the field adjacent to time entry, type in “empty cage” to specify the reason for exclusion. This annotation will appear at the corresponding excluded data in the Excluded Data File
4. Navigate back to the “Time Plots” tab and click on the “Generate plot” button
5. Click on the “Excluded data” button to download the set of automatically and manually excluded data points

Time Plots: Plot by group prior to rendering the analysis

1. To analyze only the region where the animals were maintained in the cold for analysis, use the slider bar to set the first value to 144 and the second value to 161
2. Use the “Metabolic variable” drop-down menu to select “Energy Expenditure”
3. Enter 0.75 for the “Minimum Y-value”
4. Enter 1.05 for the “Maximum Y-value”
5. Click on “Generate plot” button at the top left corner of the page. (Figure 4-3)

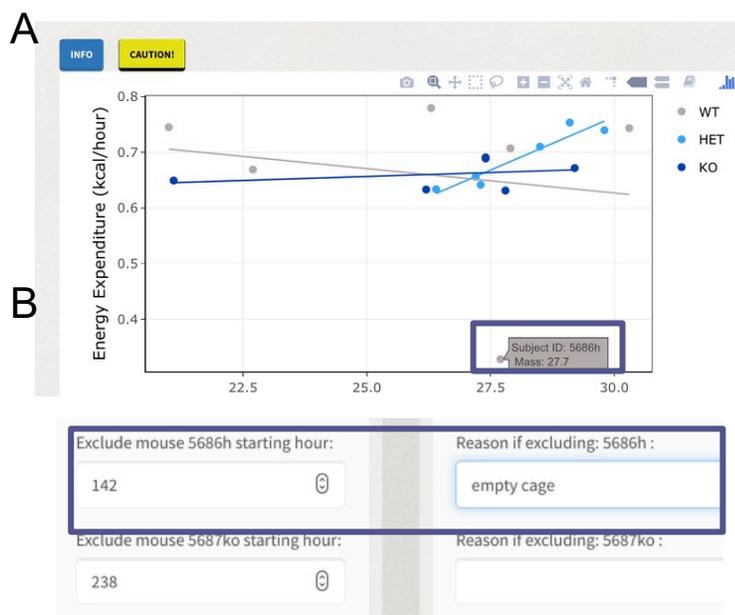


Figure 4-2. Interactive regression plots for facilitating outlier identification. (A) Hovering over the abnormal data point on the regression plot to easily determine the subject ID of the empty cage to be excluded. (B) The subject exclusion tab is used to designate the hour at which to begin excluding a cage and to provide annotation as to why the exclusion is performed.

Analysis: ANCOVA and Post hoc

To analyze the metabolic response to the cold challenge, we will select only the time region when the mice were maintained at 4°C. Once this time region is selected, the analysis tab produces the results shown in Figure 4-4.

Interpretation

We can detect that there is a significant group effect when O₂, CO₂, and EE that is a dose dependent allelic contribution. We can dig deeper to see pairwise differences from Tukey's HSD post hoc analysis. From this we can see significantly different body temperatures between some but not all groups. However, because this analysis is designed to detect an overall ordered trend, the overall group effect on EE is significant.

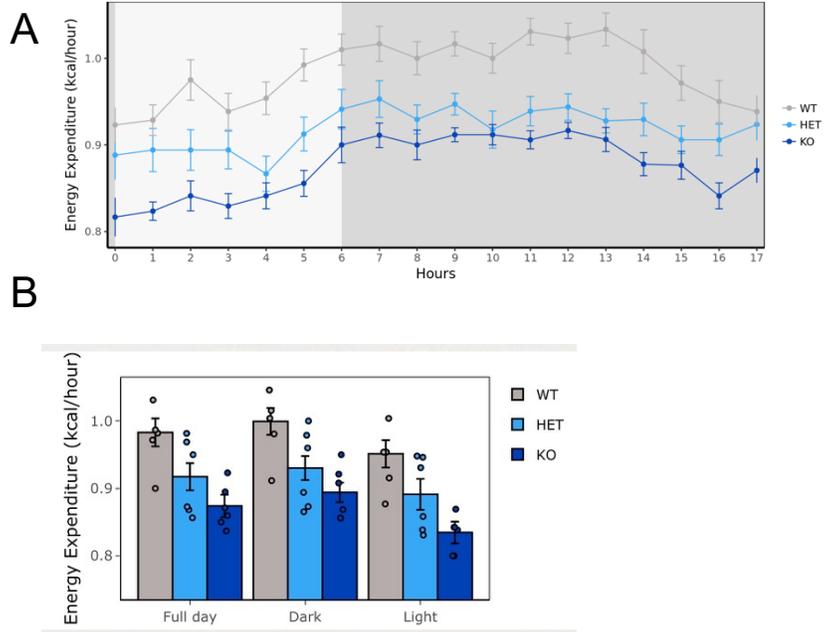


Figure 4-3. Hourly and overall averages to the three-group ordered analysis example. Prior to analysis, we select the only the period after acclimation. (A) Hourly time plot for EE (B) Overall average plot for EE at 4°C

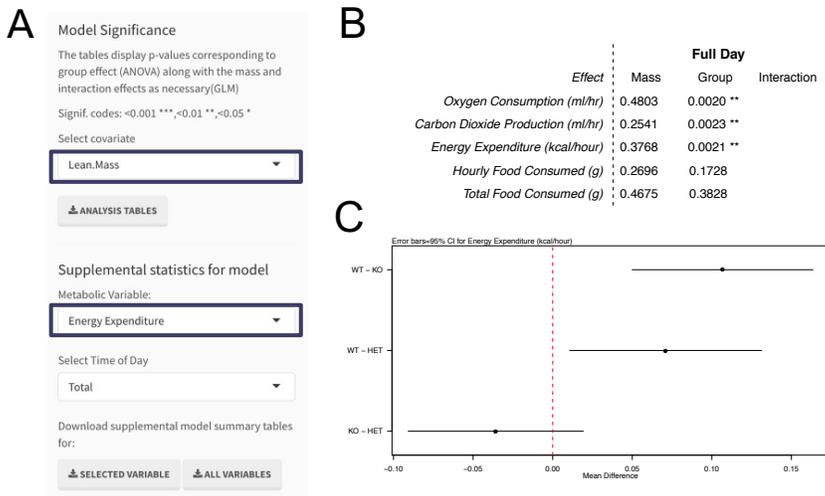


Figure 4-4. Analysis results for the three group: ordered example. (A) Selections on the Analysis Tab. (B) Analysis results. *CalR* detected no interaction effect between the three groups for any metabolic measurement, so results of an ANCOVA are returned. For the group effect, a *CalR* reports significance O₂, CO₂, and EE which should be examined by post hoc test for differences between individual groups. (C) Tukey's HSD post hoc analysis of EE shows a significant difference between wildtype and knockout groups as well as wildtype and heterozygous groups but not between knockout and heterozygous groups.

Example 5: Three-group analysis when groups are unordered

In this example, we have three unordered groups. They consist of one wild type and two unrelated knockout lines, all of the same age and genetic background. The appropriate template here is the Three-Group Analysis (non-ordered grouping). For this analysis, 19 subjects will be assigned into three groups, nine as WT, five as KO1, and five as KO2. Mice were maintained at thermoneutrality (30°C) until hour 10 of Figure 5-1 before a 3-hour transition to 4°C. The mice were maintained at 4°C at hours 13-20.

The difference between this analysis and the analysis obtained from the three groups (ordered) template is in the way the group variable is classified. In this template, our groups are factored so that the association of each knockout with the metabolic variables are computed independently of one another.

Data Input: *CaIR* and Session files

1. Under “Browse data CSV file(s)”, click on the “Browse...” button
2. Locate and open the Example5_*CaIR*.CSV file stored in the Example 5 folder
3. Under “Upload previous session inputs”, click “Browse...” and locate and open the “Example5_Session_NMR.CSV” file stored in the Example 5 folder
4. Click the “Go to plots” button to be directly navigated to the “Time Plots” tab

Time Plots: Time interval specification for each experiment

Here we are again selecting the region of maintained cold exposure for analysis (Figure 5-1).

1. The slider bar at the top of the page should have the first value at 130 and second value at 150 to view thermoneutrality, the transition to cold, and cold exposure
2. To obtain analysis for just the cold exposure experiment, set the first time value to 143 and keep the second value at 150
3. Click on Go to plots
4. Navigate to the “Analysis” tab and wait for the results to load

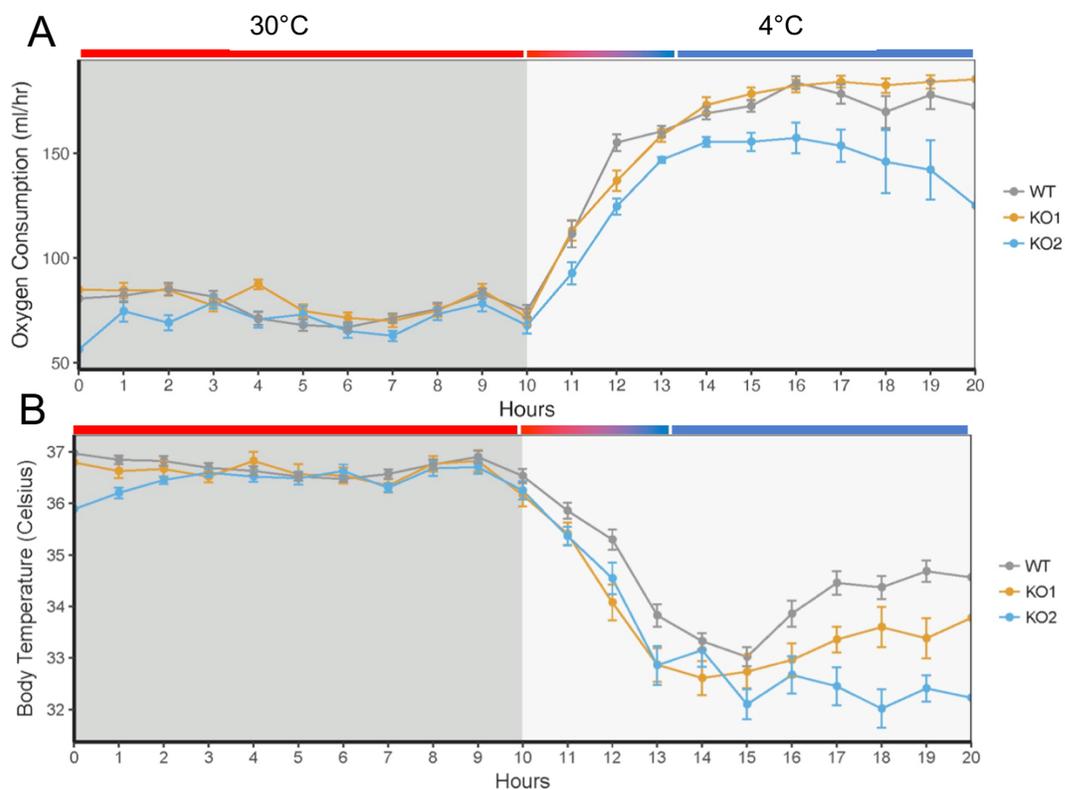


Figure 5-1. Two separate experiments examined with the Three-group: unordered analysis template. The 30°C thermoneutral experiment ends at hour 10 in the plot (red bar) and the transition to 4°C occurs between hours 10 and 13. We will select hours 13-20 to analyze the animals' response to 4°C cold challenge. (A) Oxygen consumption. (B) Body temperature.

Analysis: Unordered groups with pairwise post hoc analysis

There are several notable differences between this example and the three group: ordered analysis in Example 4. Here, an independent ANCOVA analysis is performed between the groups 1 and 2, as well as groups 1 and 3 (Figure 5-2). In contrast, in Example 4, only one overall analysis is presented. Like the Example 4 analysis, a three-way comparison is shown in the post hoc test (Figure 5-2).

1. Select “Lean.Mass” as the covariate for analysis
2. Download the resulting tables by clicking “Analysis tables”
3. By default, the post hoc plot will show oxygen consumption, but this may be changed by using the drop-down menu labeled “Metabolic Variable:”
4. Save the post hoc plot that you are viewing by clicking on the “Download this” button. Alternatively, you may download the post hoc plot for all metabolic variable by clicking “Download all”

Interpretation

Here a difference between WT and KO2 is statistically significant for VO_2 , VCO_2 , EE, and body temperature. We can visualize this significant difference by observing that the 95% confidence interval in the post hoc plot, represented by the black lines, do not cross the vertical line drawn at zero mean difference for WT and KO2 comparisons. There are no significant differences between WT and KO1 groups (Figure 5-2).

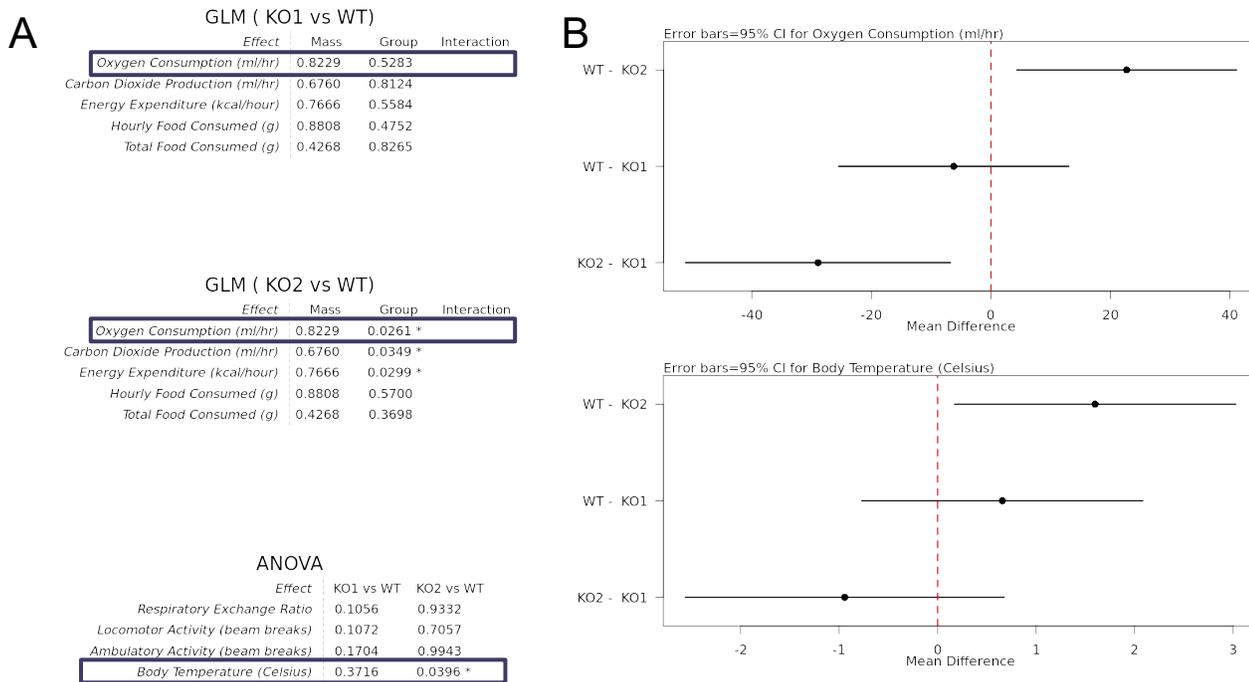


Figure 5-2. Analysis results with post hoc for the Three-group: unordered template example. (A) The ANCOVA significance values are shown in this table, which was downloaded by clicking on the “Analysis Tables” in *CalR*. (B) The mean differences in between individual groups, along with a 95% percent confidence interval, are computed using Tukey’s HSD post hoc analysis.

Example 6: Four-group analysis using pairwise comparisons and creating a CalR session file

In this example, we will demonstrate *CalR* analysis with post hoc testing for an example with four groups. This example will use 24 subjects which will be assigned to four groups when we load our session file. For this example, we will use the Four-Group Analysis template and demonstrate the steps necessary to create a session file.

The weight data collected for the subjects of this experiment contains total, fat, and lean body mass. The average group comparisons for all masses are easily observed in the bar plots under the Weight Plots tab with lines and stars automatically placed above the bars.

The analysis conducted by *CalR* will use the group variable as an unordered factored variable. For this reason, the ANCOVA/GLM results will not indicate where any group differences may arise, rather this information will be found in the post hoc results.

IMPORT INDIRECT CALORIMETER DATA

1. Browse data CSV file(s)
BROWSE... Example6_CalR.csv
Upload complete
2. Upload previous session inputs (if available)
BROWSE... No file selected
3. Designate hours for the light/dark cycle
Light cycle starts at hour (i.e. 7 for 7:00 AM):
7
Dark cycle starts at hour (i.e. 19 for 7:00 PM):
19
(Optional) Convert to standardized CalR file and download
CALR FILE

(OPTIONAL) IMPORT WEIGHT DATA*

*Weights from calorimeter will be used unless weight template is downloaded, filled out, and uploaded

1. Will you include lean and fat mass?
 Yes
 No
2. Download the template for entering subject masses
WEIGHT TEMPLATE
3. Upload filled out template form
BROWSE... Example6_weight_table_NMR.csv
Upload complete

DESIGNATE GROUPS

1. List names of groups, separated by a comma
WT Diet 1, KO Diet 1, WT Diet 2, KO Diet 2
2. Select subjects and click arrows to assign groups
a90, a92, a93, a94, a96, a98, b06, b10, b27, b28, b29, b30, c19, c20, c31, c37, c41, c42, d01, d02, d03, d13, d15, d25
9. GO TO PLOTS

12 GENERATE PLOT

13 SESSION FILE

Click to refresh plot

Save workflow

Remove outliers?
 Yes
 No

Plot by: Group

Color by: Group

Axis line width: 1

Metabolic Variable: Oxygen Consumption

Hours: 0, 14, 10, 62, 162

Download summary report: SUMMARY REPORT

Download excluded data: EXCLUDED DATA

Minimum Y-value

Maximum Y-value

Color of Group 1: #FFA6A

Color of Group 2: #A1000

Color of Group 3: #63C3F

Color of Group 4: #00599

11

Figure 6-1. Steps which can be saved into a *CalR* Session file. The Session file stores information regarding aspects of the experimental set-up including photoperiod, subject groupings, time range of interest, and colors. See text for details.

Data Input: *Ca/R* file

1. Under “Browse data CSV file(s)”, click on the “Browse...” button
2. Locate and open the Example6_*Ca/R*.csv data file stored in the Example 6 folder

Creating a *Ca/R* Session file

Our session file can be created once the following information is specified: subject identifiers (from data files), photoperiod, body composition data, group assignments, and group colors (Figure 6-1).

1. Specify the start of the light photoperiod at “6” for 0600/6AM
2. Specify the start of the dark photoperiod at “18” for 1800/6PM
3. Click “Yes” under “Will you include lean and fat mass?” Download the weight template
4. Open the downloaded file “weight_table_template.csv” with Microsoft Excel or similar software and enter Total Mass, Lean Mass and Fat Mass data from the provided file “Example6_weight_table_NMR.csv”
5. Upload the filled-out template form
6. Enter group names “WT Ctrl, WT Trt, KO Ctrl, KO Trt”
7. Specify groups
 - a. Under “Select Subjects and click arrows to assign groups” scroll down to subjects’ identifiers starting with d01. Hold down the Shift key to select the six samples d01 through d25. Click on the right arrow to move the samples into the second column. Then click the next right arrow again to move samples to the third column, repeat until samples are in the fourth column
 - b. Next select six samples c19 through c42. Click on the right arrow to move the samples into the second column. Then click the next right arrow again to move samples to the third column
 - c. Next select six samples b06 through b30. Click on the right arrow to move the samples into the second column
8. Click “Go to Plots”
9. Under “Time Plots” adjust the slider bar to select hours 14 to 62
10. Specify colors for groups 1-4 as #FFA6A6, #A10000, #63C3FF, #005994
11. Click “Generate Plot”
12. Under “Save Workflow” Click “Session File” to download the session file

Time Plots: Visualizing four groups

1. In the Time Plot tab, we set a two-day period after acclimation for analysis. We can observe the hourly averages and time of day averages for each day (Figure 6-2)
2. After confirming that this is the time period we wish to analyze, we will look at masses in the “Weight Plot” tab

Mass Plots: Pairwise comparisons

1. In the “Weight Plot” tab we are provided with two plots showing group differences in body mass (left) and body composition (right) (Figure 6-3)
2. Now we can look at metabolic variables in the “Regression Plots” tab

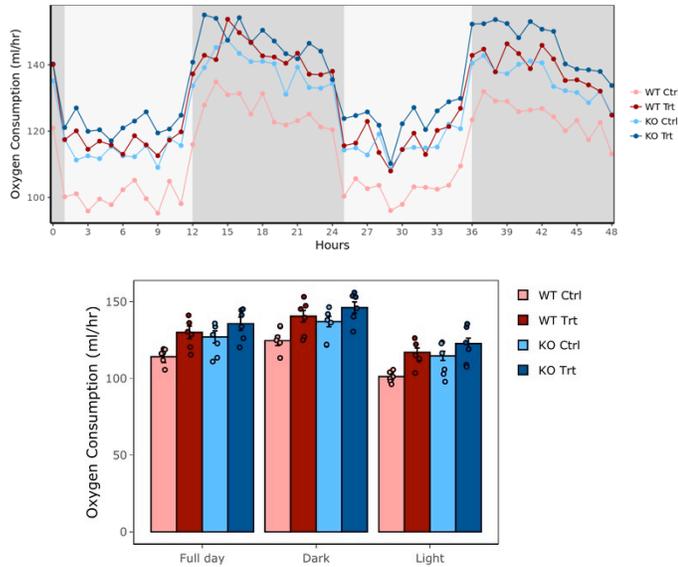


Figure 6-2. Viewing hourly and daily periodic averages for four groups.

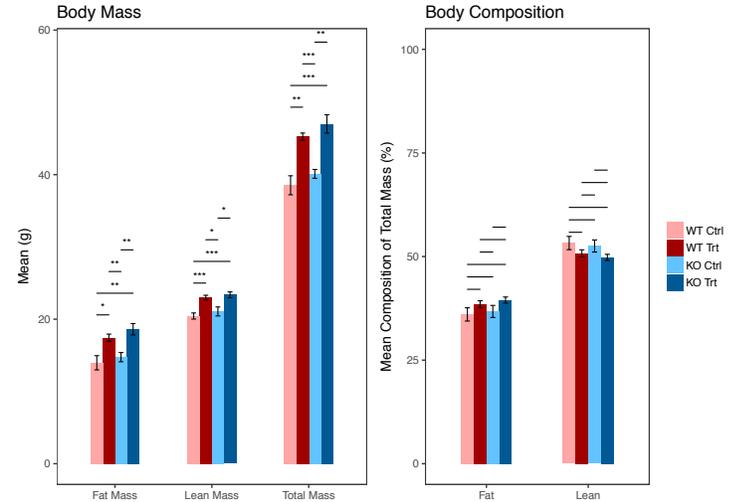


Figure 6-3. Weight plots for the Four-group analysis example. Two-sample t-tests are conducted for each group comparison denoted by the lines above the bars. Significance for each comparison is annotated by stars above the lines.

Regression Plot: Associating total body mass with EE

The 2D and 3D regression plots provide us with a visualization of each mouse's average EE plotted against their mass (Figure 6-4).

Analysis: ANCOVA and Post Hoc

The analysis tab divides the results into three GLM tables (one for each group effect against the comparator group) (Figure 6-5) and one ANOVA table for the metabolic variables that are predetermined to be body mass independent.

The post hoc plots are computed based on the GLMs to show pairwise effect sizes.

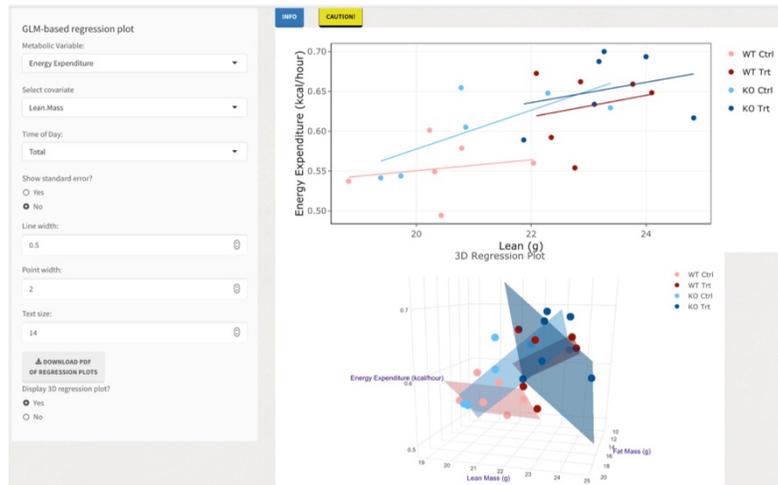


Figure 6-4. 2D and 3D regression plots for the Four-group template. In the 2D plot of Energy Expenditure vs. Lean Body Mass, we can see positive slopes which reach the threshold for statistical significance for “Mass effect” but not for “Group Effect”

A

GLM (WT Trt vs WT Ctrl)									
Effect	Mass	Full Day			Light			Dark	
		Group	Interaction	Group	Interaction	Mass	Group	Interaction	
Oxygen Consumption (ml/hr)	0.1019	0.3636		0.1157	0.3103		0.1244	0.4573	
Carbon Dioxide Production (ml/hr)	0.1384	0.2780		0.1202	0.2210		0.1971	0.3784	
Energy Expenditure (kcal/hour)	0.1172	0.3197		0.1451	0.3169		0.1380	0.3790	
Hourly Food Consumed (g)	0.2613	0.0276	0.0272	0.2586	0.0159	0.0162	0.6291	0.4195	
Total Food Consumed (g)	0.0349	0.1638		0.0298	0.1596		0.0384	0.1674	

GLM (KO Ctrl vs WT Ctrl)									
Effect	Mass	Full Day			Light			Dark	
		Group	Interaction	Group	Interaction	Mass	Group	Interaction	
Oxygen Consumption (ml/hr)	0.1019	0.1686		0.1157	0.1276		0.1244	0.2829	
Carbon Dioxide Production (ml/hr)	0.1384	0.2358		0.1202	0.1005		0.1971	0.4600	
Energy Expenditure (kcal/hour)	0.1172	0.1780		0.1451	0.1112		0.1380	0.3131	
Hourly Food Consumed (g)	0.2613	0.8193	0.8117	0.2586	0.7809	0.7612	0.6291	0.7832	
Total Food Consumed (g)	0.0349	0.9509		0.0298	0.8356		0.0384	0.9797	

GLM (KO Trt vs WT Ctrl)									
Effect	Mass	Full Day			Light			Dark	
		Group	Interaction	Group	Interaction	Mass	Group	Interaction	
Oxygen Consumption (ml/hr)	0.1019	0.1219		0.1157	0.1134		0.1244	0.1676	
Carbon Dioxide Production (ml/hr)	0.1384	0.0443		0.1202	0.0439		0.1971	0.0680	
Energy Expenditure (kcal/hour)	0.1172	0.0791		0.1451	0.0926		0.1380	0.1033	
Hourly Food Consumed (g)	0.2613	0.2756	0.2482	0.2586	0.1987	0.1837	0.6291	0.3486	
Total Food Consumed (g)	0.0349	0.3087		0.0298	0.2908		0.0384	0.3199	

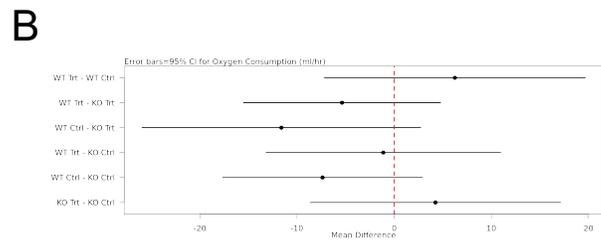


Figure 6-5. Analysis of the Four-group analysis template example. (A) The GLM shows us that after accounting for differences in lean weight, by setting it as a covariate, we do not observe any statistically significant differences between the groups in any of the metabolic variables recorded. (B) We can observe all six pairwise comparisons in the post hoc plots.

Example 7: Crossover study analysis

Investigators may wish to utilize a crossover study to have each individual serve as their own control when administering two treatments. This type of experiment is less common due to a fear of carryover effects as the first treatment may not be completely washed out by the second treatment. *CalR* implements a rigorous analysis to test for potential carryover effects.

First, we will upload the calorimetry data, which contains the recordings from both treatments periods within the same file (Figure 7-1). After that, we will upload the session file, which contains the group specifications based on the order in which they received the treatments (i.e., Veh-Drug corresponds to the subjects receiving vehicle treatment first and drug second).

Next, we will specify the periods during which treatment responses were observed. In the time plots tab, there will be two plots with corresponding slider bars. The upper slider and plot will correspond to the first treatment period and the lower slider and plot will correspond to the second treatment period.

Lastly, the analysis tab will inform us of any significant difference between the treatments. The period effect is presented because it accounts for any differences attributed to the distinct times of treatment administration. Since there is a possibility that there was inadequate washout time, *CalR* also tests for an interaction between the period and treatment effects.

Data Input: *CalR* and Session files

1. Under “Browse data CSV file(s)”, click on the “Browse...” button
2. Locate and open the Example7_*CalR*.CSV file stored in the Example 7 folder
3. Under “Upload previous session inputs”, click “Browse...” and locate and open the “Example7_Session_NMR.CSV” file stored in the Example 7 folder
4. Click the “Go to plots” button to be directly navigated to the “Time Plots” tab (Figure 7-1)

Figure 7-1. Input tab for Crossover Analysis. The *CalR* file uploaded contains measurements from both treatment periods. Group names should specify the order in which each group received the treatments. This information, along with the hours of the two treatment periods is contained within the session file.

Time Plots: Select time ranges

1. The slider bar at the top of the page should have the first value at 70 and second value at 73
2. The slider bar below the first plot should have the first value at 94 and second value at 97
3. Click on “Generate plot” button at the top left corner of the page (Figure 7-2)

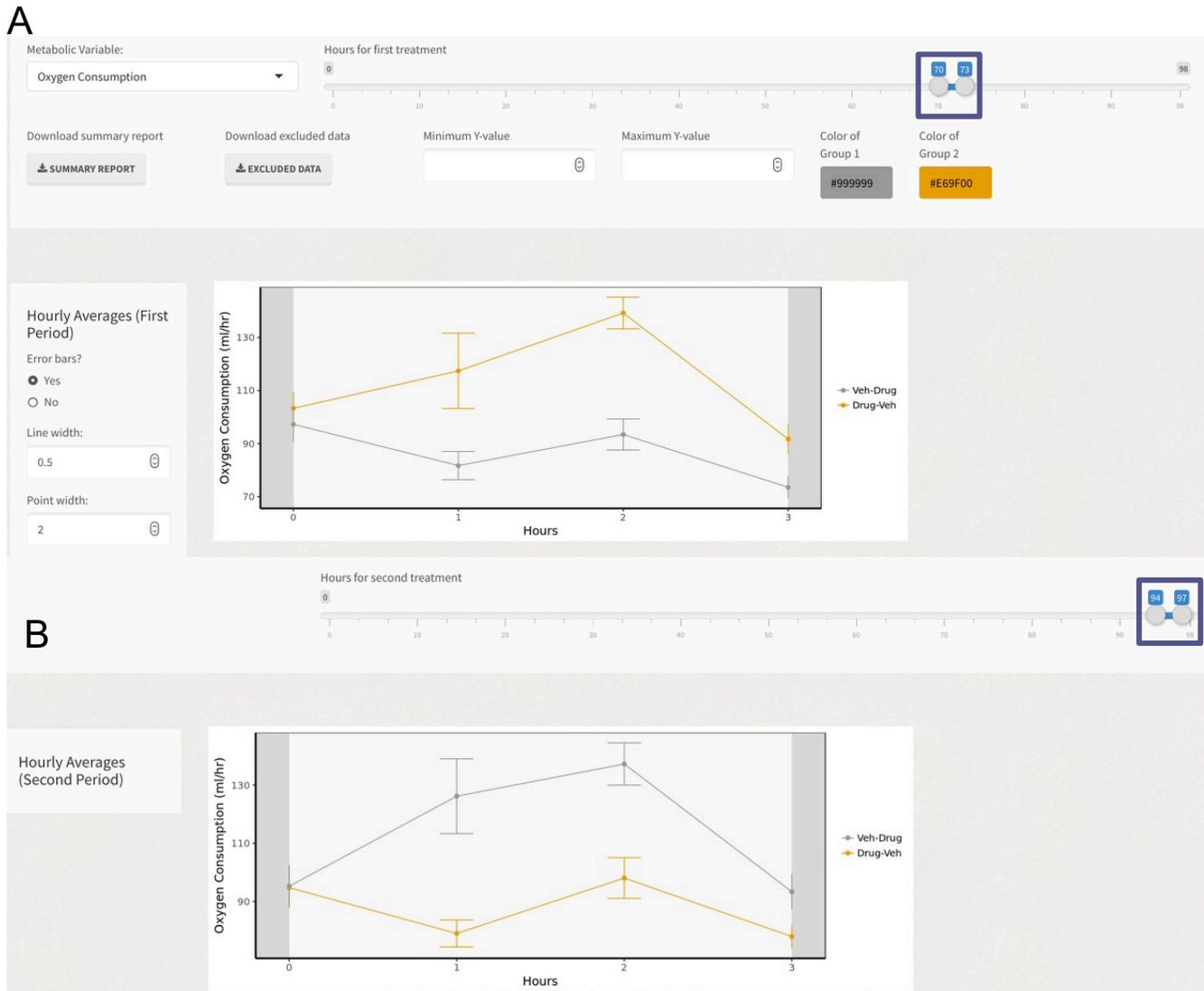


Figure 7-2. Time Plots tab for Crossover Template. (A) The upper slider bar sets to the hours during which the subjects are responding to the treatment administered first. (B) The lower slider bar sets the hours during which the subjects are responding to the treatment administered second.

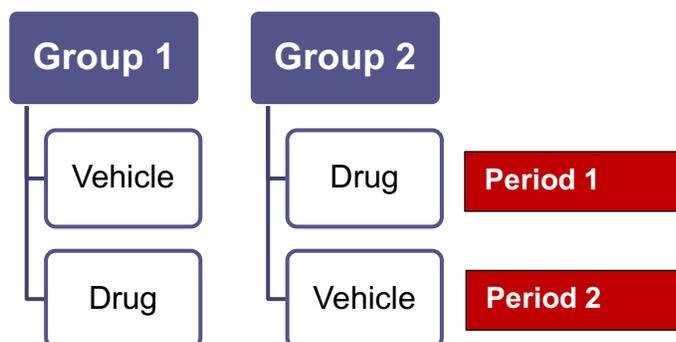
Analysis: GLM

- The analysis tab divides the results into two GLM tables, one for mass-dependent variables and the other for mass-independent variables (Figure 7-3).
- The treatment effect compares the first group’s measurements from the first period together with the second group’s measurements from the second period with the first group’s measurements from the second period together with

the second group's measurements from the first period (e.g. Vehicle_{Grp1} and Vehicle_{Grp2} versus Drug_{Grp1} and Drug_{Grp2}) (Figure 7-3).

- The period effect tests whether the different time of treatment influenced the metabolic variables.
- The interaction effect between period and treatment tests whether there is a carryover effect from the first set of treatments onto the second one.

A



B

Model Significance

The tables display p-values corresponding to group effect (ANOVA) along with the mass and interaction effects as necessary (GLM)

Signif. codes: <0.001 ***, <0.01 **, <0.05 *

Select covariate

Lean.Mass

ANALYSIS TABLES

Supplemental statistics for model

Metabolic Variable:

Oxygen Consumption

Select Time of Day

Total

Download supplemental model summary tables for:

SELECTED VARIABLE **ALL VARIABLES**

INFO

GLM (mass-dependent)

Effect	Mass	Ttrtmnt	Period	Interxn (Mass/Ttrtmnt)	Interxn (Period/Ttrtmnt)
Oxygen Consumption (ml/hr)	0.1044	<0.001 ***	0.8411		
Carbon Dioxide Production (ml/hr)	0.0428 *	<0.001 ***	0.9855		
Energy Expenditure (kcal/hour)	0.0846	<0.001 ***	0.8731		
Hourly Food Consumed (g)	0.8224	0.2545	0.2112		
Total Food Consumed (g)	0.4924	0.3922	0.0324 *		
Hourly Water Consumed (ml)	0.6897	0.6686	0.6585		
Total Water Consumed (ml)	0.9111	0.0616	<0.001 ***		

GLM (mass-independent)

Effect	Ttrtmnt	Period	Interxn (Period/Ttrtmnt)
Respiratory Exchange Ratio	<0.001 ***	0.0868	
Locomotor Activity (beam breaks)	0.2456	<0.001 ***	
Pedestrian Locomotion (m)	0.9171	<0.001 ***	
Total Distance in Cage (m)	0.7751	<0.001 ***	

Mass effect: Lean.Mass

Signif. codes: <0.001 ***, <0.01 **, <0.05 *

Figure 7-3. Analysis scheme and results for Crossover Template. (A) Two group crossover experimental design for two treatments given at alternating time periods. (B) The “GLM (mass-dependent)” table shows that the treatment significantly affected energy expenditure. We observe a significant period effect for total food consumed and total water consumed, which is intuitive since the subjects would have consumed more total food and water between the first and second periods. Fortunately, there is no interaction between treatment and period, indicating that there was an adequate washout of the treatments between the two periods.

Example 8: Combining two experimental runs

Experimental realities dictate that sometimes more than one experimental run must be combined. We often experience this because there are insufficient metabolic cages available to accommodate a large cohort of animals (i.e., too many mice per run). Alternatively, getting sufficient numbers of well-matched littermate controls for genetically modified mouse lines can mean combining the data from multiple cohorts (too few mice per run). Also, an investigator may deliberately run a group of animals more than once to compare the effect before and after an intervention. Regardless of the motivation, each instance of combining runs must be carefully considered for experimental validity as there are significant caveats which exist. These concerns include attention to calibration effect, differences in animal group characteristics (e.g., age), or seasonal effects. As in all calorimetry analysis, an instrument which has been incompletely calibrated will produce spurious results which, if combined with a correctly calibrated run will lead to invalid analyses. Age and body weight are critical factors influencing metabolism. Efforts should be made to use animals which are as closely matched as possible. Time is also a critical factor in metabolic studies, consequently, *CalR* will align each run to the clock time to ensure that runs are made with maximum comparability. Seasonal differences may also affect mouse metabolism, despite housing in a light, temperature, and humidity controlled vivarium. We encourage investigators to report whether one or more runs were combined when describing their experimental methods and any factors which may be relevant to the interpretation.

This example uses the Combined Run template. In both Run 1 and Run 2, the animals were administered a sample compound or saline control. In this example, we will combine two runs, each with six animals (n=3 Saline and n=3 Drug-treated). The data files from the two runs will be combined into a single *CalR* file which we will analyze with the two-group template as in Example 1.

To combine more than two runs, the merged *CalR* data file from the two runs can be re-loaded into this template and combined with additional data files to merge three or more runs where appropriate. Note that this template will modify subject identifiers requiring updated subject identifiers in corresponding weight tables or *CalR* session files.

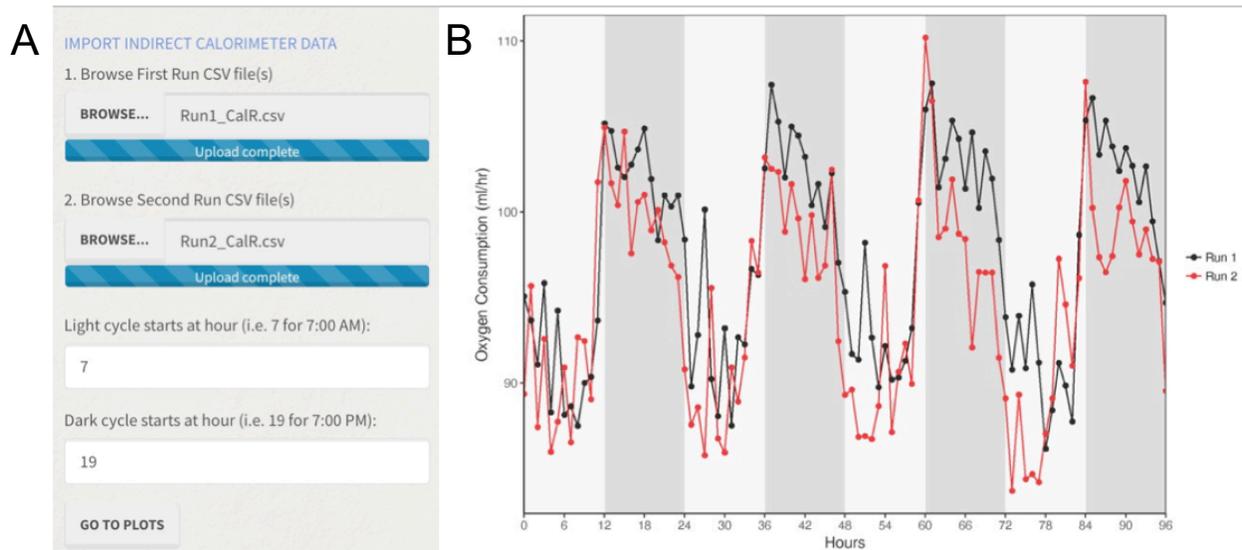


Figure 8-1. Example of the *CalR* Combined Run Template (A) The Input menu for the combine two runs template. (B) Time plot of all mice in Run 1 and Run 2. This view does not consider group or genotype during alignment.

In this experiment, there were only six metabolic chambers available for 12 mice, and two successive runs were necessary. These two runs were performed within one week with the calorimeter was operating under the same calibration standards.

Data Input: *CalR* files

1. Under “Browse First Run CSV file(s)”, click on the “Browse...” button.
2. Locate and open the Run1_*CalR*.CSV file stored in the Example 8 folder
3. Under “Browse Second Run CSV file(s)”, click on the “Browse...” button.
4. Locate and open the Run2_*CalR*.CSV file stored in the Example 8 folder
5. Click “Go to Plots” button
6. Click “Generate Plots” button
7. Adjust the number of hours or days to offset (if necessary)
8. Click Download Combined *CalR* File (Figure 8-1)

Analysis of combined runs may be done using one of the *CalR* analysis templates. This experiment can be analyzed with the two-group template using the provided session file “Combined_*CalR*_Session.CSV.” Following two group analysis we observed that while there were no significant differences observed in either Run 1 or Run 2 with n=3 animals per group each, when combined (n=6 animals per group), multiple parameters reach statistical significance (Figure 8-2).

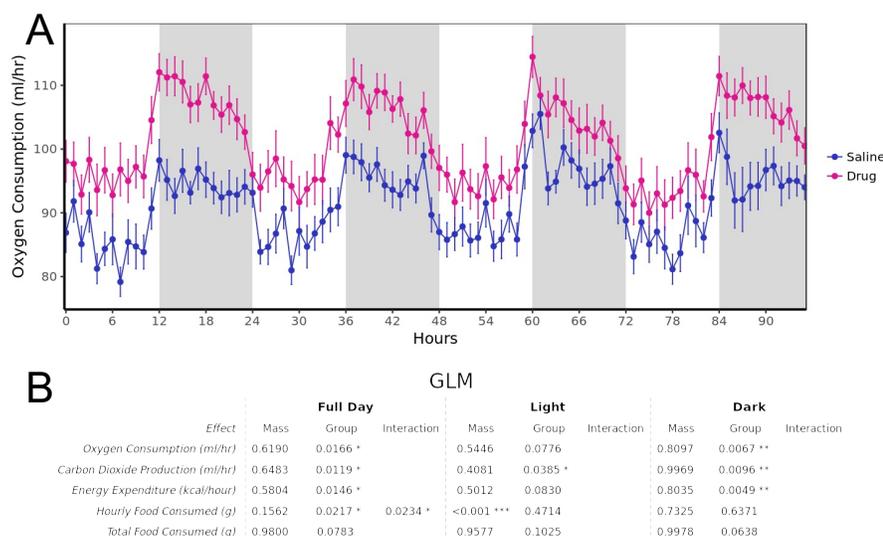


Figure 8-2. Following the combination of Run 1 and Run 2, a new *CalR* data file was created. Analysis was subsequently performed using the 2-group analysis template. (A) Time plot of VO_2 . (B) GLM analysis table.